Clinical and Experimental Allergy, 1995, Volume 25, pages 828–838

Bee venom immunotherapy induces a shift in cytokine responses from a TH-2 to a TH-1 dominant pattern: comparison of rush and conventional immunotherapy

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Summary

Background The mechanism of immunotherapy is unclear. Allergic disease is known to involve enhanced TH-2 cytokine responses to allergen.

Objective In order to investigate the mechanisms of immunotherapy, we have examined changes in cytokine secretion before (13 patients) and during (nine patients) both rush and conventional venom immunotherapy (VIT) in bee venom allergic patients.

Methods Peripheral blood mononuclear cells were stimulated *in vitro* with bee venom, non-specific antigen or mitogen and secretion of IL-4 (TH-2) and IFNγ (TH-1) over the culture period measured.

Results Untreated patients had TH-2 responses to venom and TH-1 responses to antigen and strong proliferative responses to venom. Controls showed no response (proliferation or cytokines) to venom and the normal TH-1 response to antigen. VIT resulted in marked changes in cytokine secretion to venom, with reduction of the abnormal TH-2 response and induction of a TH-1 response. The pattern differed in rush and conventional VIT. One day after rush VIT there was a significant fall in IL-4 secretion ($P < 0{\cdot}01$), which rose by 3 weeks then declined. In conventional VIT there was a gradual reduction of IL-4 production significant after 2 months and undetectable by 6 months. IFNγ secretion was induced by VIT. Proliferative responses mirrored the IL-4 changes. One day after rush VIT there was a loss of T cells, monocytes and NK cells from peripheral blood.

Conclusion This study shows that immunotherapy shifted cytokine responses to allergen from a TH-2 to a TH-1 dominant pattern, suggesting direct effects on T cells. How these cytokine changes relate to clinical desensitization is not clear. In the longer term they would result in an isotype switch from IgE to IgG. Early changes in cytokine or chemokine production might downregulate mast cell or basophil reactivity and explain the rapid desensitization in rush VIT.

Keywords: immunotherapy, mechanism, bee venom allergy, cytokines, TH-1 and TH-2 responses

Clinical and Experimental Allergy, Vol. 25, pp. 828–838. Submitted 1 August 1994; revised 14 February 1995; accepted 3 March 1995.

Introduction

Venom immunotherapy (VIT) with pure venom extracts is highly effective and therefore provides a good model for the study of mechanisms of immunotherapy. The mechanism(s) is unclear, but the main hypothesis advanced is the generation of specific IgG (blocking) antibody. IgG levels have been shown to increase during conventional immunotherapy (IT) for both venom and inhalant allergens, particularly with increases in IgG1 and

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later in IgG4 subclasses [1–3]. It has been postulated that this increase is responsible for competitive inhibition of allergen-IgE interactions on the mast cell. In the case of venom allergy, this concept was supported by observations that protection from bee stings could be passively transferred with hyperimmune serum from beekeepers [4]. In addition, VIT which would otherwise have been abandoned because of systemic reactions, could be continued following infusion of hyperimmune serum [5,6]. However, a considerable body of evidence [7–10] suggests that there is no correlation between total venom-specific IgG or IgG subclass levels and reduced sensitivity to stings. It remains possible that relative changes in the ratio of IgG to IgE to individual components of venom may be involved in the mechanism, but this is difficult to quantify.

In recent years considerable advances have been made in the understanding of the control mechanisms of IgE isotype selection and IgE production. The central role of the cytokines, interferon-gamma (IFNγ) and interleukin 4 (IL-4) in IgE regulation has been established, and their preferential expression by two distinct T helper cell populations (TH-1 and TH-2 cells respectively) demonstrated in mice and also in man [11–13]. As IgE is central to allergic hypersensitivity, down-regulation of IgE production may be an important component of the mechanism of IT. However, this explanation may be too simplistic since although specific IgE levels in serum fall with IT in the long-term, these are usually increased initially and at the time clinical desensitization is achieved, in conventional IT [1]. Allergic subjects have been shown to have enhanced TH-2 (IL-4) responses and impaired TH-1 (IFNγ) responses to specific allergens. Thus, allergen-specific T cell clones (TCC) from patients allergic to *Dermatophagoides pteronyssinus* secrete IL-4 but little or no IFNγ, whereas TCC directed against non-allergenic antigens from the same subjects secrete IFNγ [14]. Similarly, using a model we have developed to study the cytokine secreting profile of peripheral blood mononuclear cells (PBMC) *in vitro*, we have shown house dust mite allergic patients have a TH-2 response to simulation with allergen, but a normal TH-1 response to stimulation with a recall non-allergenic antigen [15,16].

The aim of this study was to determine whether rush or conventional venom IT led to a change in cytokine secretion, with a switch from TH-2 to TH-1 responsiveness to specific allergen. Here we report changes in cytokine profiles in culture supernatants from PBMC stimulated *in vitro*. The production of IL-4 and IFNγ was measured, after *in vitro* stimulation with the T cell mitogen, phytohaemaglutinin (PHA), an ubiquitous recall antigen, streptokinase-streptodornase (SK-SD) and bee venom (specific allergen) in venom allergic patients during VIT. Before treatment patients showed a TH-2 response to venom and a normal TH-1 response to non-specific antigen. VIT led to a switch from IL-4 (TH-2) to IFNγ and IL-2 (TH-1) production. The pattern of cytokine change differed in rush and conventional VIT. In rush VIT a marked reduction in IL-4 secretion by PBMC occurred after 1–2 days whereas in conventional VIT a more gradual loss of IL-4 secretion occurred. Possible mechanisms of desensitization are discussed.

Materials and methods

Patients were selected for VIT on the basis of a history of severe systemic reactions to bee stings. Sensitivity was confirmed by intradermal skin tests with bee venom and measurement of serum specific IgE antibodies by radioallergosorbent test (CAP/RAST). Thirteen bee venom allergic patients were studied before treatment and nine of these during honey bee venom (*Apis mellifera*) IT. In five patients (two male and three female) a rush VIT regime was used and in four patients (two male and two female) a conventional VIT regime was used. The rush regime was used to minimize the number of outpatient visits or if rapid induction of desensitization was required, so that no particular subgroup of patients was selected for this treatment. As a control group, six normal non-atopic subjects not allergic to bee venom (three males and three females) were studied to provide baseline cytokine production levels.

Immunotherapy protocols

The protocol for conventional VIT was as follows. The initial course consisted of weekly injections of pure venom (our own preparation) given over about 3 months, starting at 2 μg and with a planned top dose of 100 μg, equivalent to about two stings. The regime was modified if there was a history of reactions and the top dose was sometimes less than 100 μg. Thereafter, maintenance injections of the top dose were given 3 $\times$ monthly and then 3-monthly. In rush induction the top dose was reached in 1 or 2 days. Incremental injections of venom were given subcutaneously at 15–30 min over a few hours on 1 or 2 days. The initial dose was equivalent to 1/1000th of a sting and the top dose after the induction phase was usually between 70 and 100 μg. The cumulative induction dose was approximately 300 μg venom. Thereafter weekly injections were given for 3 weeks (incremental to 100 μg if this dose had not been reached), followed by monthly maintenance injections of the same dose 3 $\times$, then 3-monthly injections.

Establishment of cell cultures and generation of culture supernatants

PBMC were separated from heparinized whole blood by density gradient sedimentation and established in culture, as previously described [15,17]. Briefly, $0{\cdot}5 \times 10^6$ PBMC per 1 mL culture were stimulated with the T cell mitogen, phytohaemaglutinin PHA-L (lectin from Phaseolus vulgaris, Sigma) (10 μg/mL), a recall antigen steptokinase-streptodornase (SK-SD Lederle Laboratories, Gosport, UK) (10 μg/mL) and pure bee venom (3 μg/mL) (our own preparation). The doses used induced maximal responses in proliferation assays [17] and were confirmed retrospectively for each patient. The bee venom was collected by electrical stimulation, directly into diluent in a sterile closed chamber. It was characterized and standardized as previously described [10] including assessment of phospholipase A_2 activity and mellitin activity by haemolytic assays and RAST inhibition. It contains all major bee venom allergens on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), identical to the Pharmalgen bee venom preparation. The same batch of pure bee venom was used for treatment and for *in vitro* studies. All stimulating reagents were dissolved in tissue culture medium and filtered before use (0·2 μm pore size). The culture medium used in PBMC assays was RPMI-1640 (Flow Laboratories. Irvine, UK) supplemented with 100 U/mL penicillin (Sigma Chemical Co Ltd, Poole, UK), 100 μg/mL streptomycin (Sigma), 2 mM *L*-glutamine (Sigma), 20 mM N-2- hydroxyethylpiperazine-N′-2 ethanesulphonic acid (HEPES, Merck Ltd, Poole, UK) (standard RPMI) and 10% heat-inactivated, defatted, 0·2 μm filtered AB + normal human serum (Blood Transfusion Service, Addenbrookes Hospital NHS Trust). Two culture systems were set up, one involving the use of a 96 well microtitre plate to examine dose-response relationships, the other a 100 μL assay for the generation of supernatants for use in time course studies. From the first assay proliferative responses were assessed by a 6 h pulse with tritiated thymidine as previously described [17]; from the second assay where optimum doses of stimulant had been previously determined, multiple cytokine measurements could be made. Culture supernatants were harvested at time zero and every 24 h thereafter up to a maximum of 264 h and stored at −30°C before cytokines were assayed.

Assays used to determine TH-1/TH-2 cytokine production.

Inhibition ELISA for IFNγ Ninety-six well micro-ELISA plates (Dynatech Immulon M129B) were coated with 0·2 μg/mL recombinant human IFNγ (specific activity $1{\cdot}3 \times 10^6$ U/mL, 100 μg/mL; Biogen) in carbonate-bicarbonate buffer, pH 9·6, overnight at 4°C, using a working volume of 100 μL [18]. Plates were washed three times with phosphate-buffered saline containing 0·05% Tween 20 (PBS-T) and blocked with 200 μL 5% heat-inactivated fetal calf serum (FCS) in PBS-T for 1 h at room temperature. After one wash with PBS-T, 50 μL aliquots of standards (recombinant IFNγ diluted in culture medium) and samples (culture supernatants) were added, immediately followed by 50 μL/well of a 1 : 150 000 dilution (approximately 6·7 ng/mL) of monoclonal anti-human IFNγ (20-G7 a gift from Celltech) in PBS-T + 5% FCS, with the relevant controls. The plates were shaken to mix the well contents and left overnight at 4°C. After three washes with PBS-T, 100 μL/well of goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma) diluted 1 : 600 in PBS-T + 5% FCS, were added and incubated for 4 h at room temperature. Plates were washed twice with PBS-T and once with distilled water and 100 μL/well of the enzyme substrate para-nitrophenyl phosphate at 1mg/mL in diethanolamine buffer (pH 9·8) were added and the colour allowed to develop for 2 h at 37°C in the dark. Plates were read at 405 nm in an automated plate reader (BIO-RAD 3550). Results were analysed using Microplate Manager software (BIO-RAD). The lower level of sensitivity of this assay was between 0·03 and 0·1 ng/mL. This assay has been extensively calibrated and compared across a panel of different antibodies [18].

Indirect sandwich ELISA for IL-4 Ninety-six well micro-ELISA plates (Dynatech Immulon M129B) were coated with 0·5 μg/mL murine monoclonal anti-human IL-4 (Cambio, Cambridge, UK) in carbonate-bicarbonate buffer, pH 9·6, overnight at 4°C, using a working volume of 100 μL. Plates were washed three times with PBS-T and blocked with 150 μL 5% heat-inactivated FCS in PBS-T for 1 h at room temperature. After one wash with PBS-T, 100 μL aliquots of standards (recombinant human IL-4; British Biotechnology Ltd, Oxford, UK) and samples (culture supernatants) with the relevant controls were added. The plates were shaken to ensure even distribution of the well contents and left overnight at 4°C. After three washes with PBS-T, 100 μL/well of polyclonal goat anti-human IL-4 (Sigma) diluted to 2·5 μg/mL in PBS-T + 5% FCS, were added and incubated for 2 h at room temperature. Plates were washed three times and 100 μL/well of rabbit anti-goat IgG conjugated with alkaline phosphatase (Sigma), diluted 1 : 500 in PBS-T + 5% FCS, were added and incubated for a further 2 h at room temperature. After three final washes, two with PBS-T and one with

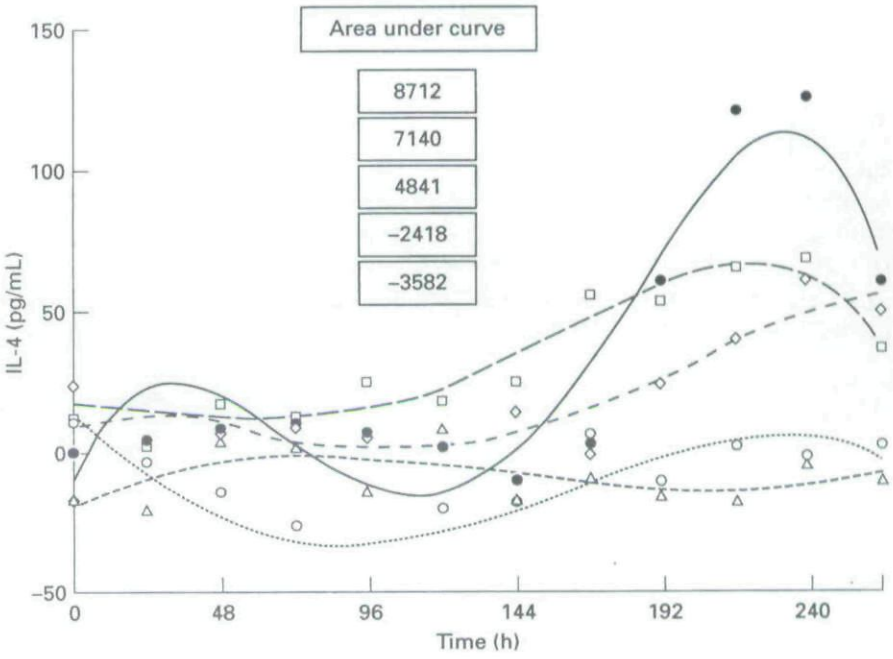

Fig. 1. IL-4 production by a bee venom allergic patient during conventional bee venom IT. Representative figure showing IL-4 production by PBMC at 24 h intervals in response to stimulation with 10 μg/mL bee venom, measured by IL-4 ELISA. The curves represent 4th order polynomial fits of the data from cultures started before (pre) and during VIT as shown on the key. IL-4 production over time was calculated as the area under each curve for bee venom stimulation, with control values derived from parallel unstimulated cultures already subtracted. This patient was studied on five occasions before and during VIT. The co-efficient of variation between the control levels for each of the five studies was 12·7%, with a mean of 8637 pg/mL (representing control IL-4 production and assay background). –●–. Pre. –□–. +3 weeks. –◇–. +2 months. –△–. +3 months. ···○···. +4 months.

distilled water, 100 μL/well of the enzyme substrate para-nitrophenyl phosphate (Sigma) at 1 mg/mL in diethanolamine buffer (pH 9·8) were added and the colour allowed to develop for up to 2 h at 37°C in the dark. Plates were read at 405 nm in an automated plate reader (BIO-RAD 3550). Results were analysed using Microplate Manager software (BIO-RAD). The lower level of sensitivity of this assay was 1·0–3·0 pg/mL.

Cell numbers

The yield of PBMC per mL of blood was calulated before and 24 h after the start of rush VIT. The number of T cells, T cell subsets, monocytes and NK cells were measured using the appopriate monoclonal antibodies and FACS analysis.

Analysis and statistics

The *in vitro* culture system and subsequent assays provide antigen-specific kinetic profiles for each cytokine measured. These profiles are generally characterized by a lag phase followed by induction of production. This peaks and subsequently declines as consumption and/or degradation occurs (Fig. 1). The profiles varied between subjects and for different stimuli, both in terms of peak levels detected, shape of the curve and the time of maximum response. In the case of IL-4 production to bee venom stimulation in patients, the time to reach the peak varied from 144 to 240 h. Taking the peak level of the cytokine production at a single time point could be unrepresentative. Because of this variation, we adopted the area under the curve analysis. The total amount of cytokine detected over the culture period (a) was calculated by applying a curve of best fit (4th order polynomial) to the data and integrating to obtain the area under the curve (Fig. 1). In order to normalize the results for statistical comparison the derived figure was divided by cytokine production detected in a parallel unstimulated culture (b). Where appropriate, the results are expressed as a proportional increase (or decrease) compared with the control, calculated by the formula (a/b–1). Group means were analysed using the Student's *t*-test.

Results

In order to establish a baseline against which the effects of bee venom immunotherapy could be measured we assessed the cytokine production profiles and T cell proliferative responses to bee venom in untreated bee venom allergic subjects and non-allergic controls. There was a strong proliferative response in the sensitive subjects indicating that bee venom-specific T cells are present in the circulation of these individuals, but not in controls (Fig. 2). Both bee venom-sensitive subjects and controls elaborated a TH-1 type profile when PBMC were stimulated with the recall antigen SK-SD, characterized by production of high levels of IFNγ but no IL-4. However, only the bee venom sensitive subjects

produced IL-4 after specific allergen challenge *in vitro* confirming that their response to bee venom was predominantly TH-2 (Fig. 3).

In order to establish repeatability of these baseline observations on cytokine secretion, we studied five subjects (two untreated patients, and three controls) repeatedly. Using paired-readings for IL-4 and IFNγ production in response to PHA stimulation for five subjects, a coefficient of repeatability was calculated, as described by Bland and Altman [19]. Repeatability (defined as 95% of the differences between the two observations being less than 2 standard deviations) was demonstrated, when comparing either peak cytokine production on two occasions or paired readings of cytokine production at each period of culture (e.g. 1, 2, 3 days etc.).

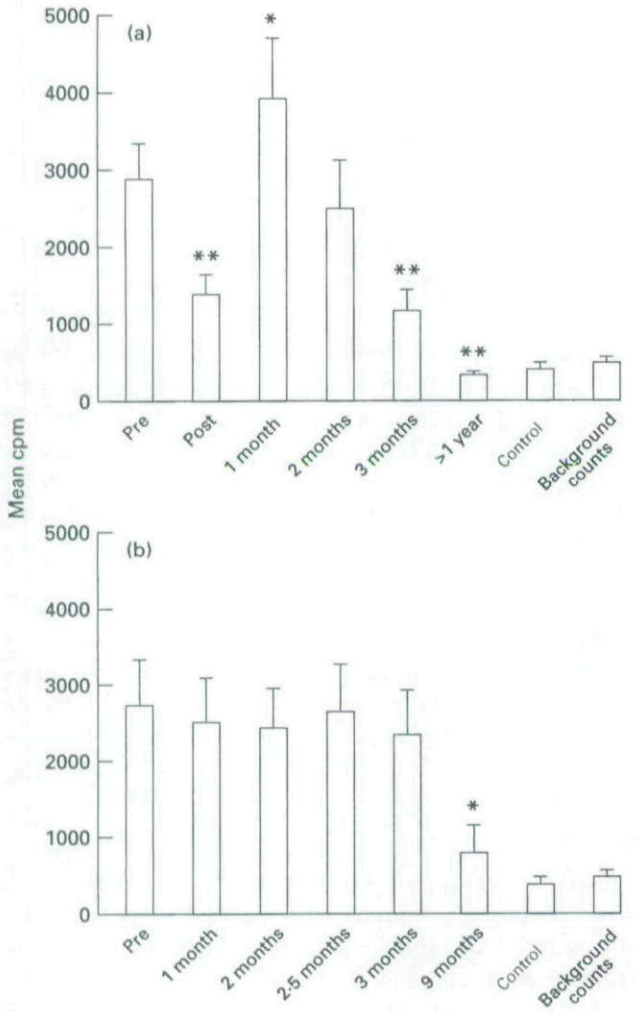

Fig. 2. Proliferative responses to bee venom during bee venom immunotherapy. Combined PBMC proliferative responses (mean ± SE) to a range of concentration of bee venom (10 μg/mL – 0·1 μg/mL in half log dilutions) during (a) rush IT ($n=5$, top graph) and (b) conventional IT ($n=4$). Statistical significance from pre-IT levels is indicated: $P<0{\cdot}01$ (**) or $P<0{\cdot}05$ (*). Proliferation by non-sensitive controls ($n=6$, control) and background counts are shown.

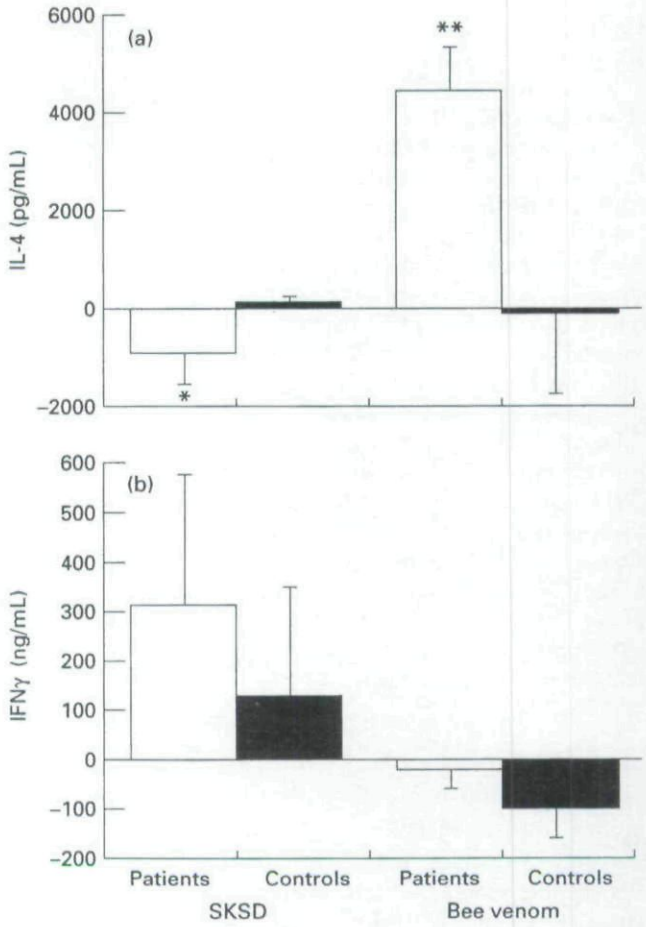

Fig. 3. IL-4 and IFNγ production by PBMC of untreated bee venom sensitive patients and controls. Histograms (means ± SE) showing (a) IL-4 production and (b) IFNγ production by PBMC in response to stimulation with SKSD (recall antigen) and bee venom in venom-sensitive patients ($n=13$) and non-sensitive controls ($n=6$). Statistically significant increases or decreases compared with unstimulated cells is indicated: $P<0{\cdot}01$ (**) or $P<0{\cdot}05$ (*). All subjects mount a predominantly TH-1 response to SKSD (IFNγ but no IL-4), whereas only patients mount a TH-2 response to bee venom (IL-4 but no IFNγ).

The T cell proliferative response to bee venom during VIT differed between conventional and rush protocols. In rush IT there was a marked fall in proliferation after the induction phase (at 24 h). This recovered to pre-treatment levels by 1 month but showed a significant reduction after 3 months; after 1 year there was no proliferative response to bee venom (Fig. 2). During

conventional VIT the response remained relatively unchanged until after 3 months treatment (i.e. until the end of the induction phase) whereafter it declined (Fig. 2). Controls had no proliferative response to bee venom (Fig. 2) but both sensitive and non-sensitive subjects exhibited normal responses to recall antigen and mitogen stimulation (data not shown).

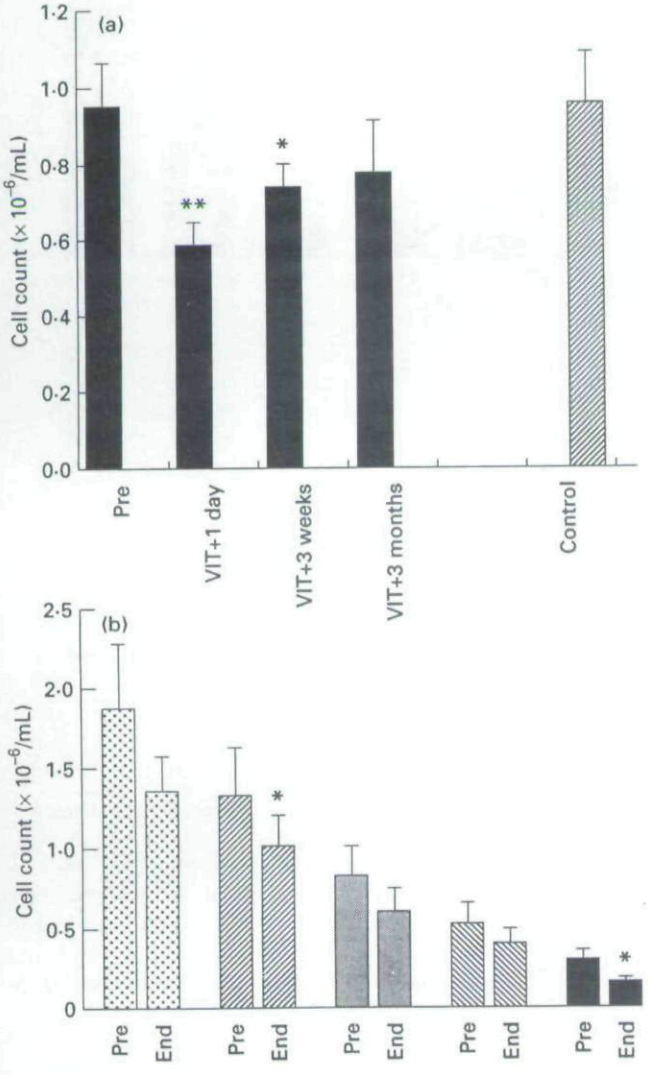

Fig. 4. Reduction in mononuclear cell recovery during induction phase of rush VIT. Graphs showing significant reduction in PBMC recoveries (a) and leucocyte subsets (b) following the induction phase of rush VIT in 5 patients (means ± SE). (a) Statistically significant decreases compared with pre-IT levels are indicated on graph: $P < 0{\cdot}01$ (**) or $P < 0{\cdot}05$ (*). The usual range for PBMC recovery in represented by six controls. (b) The % changes in cell numbers from before (pre) to the end of the induction phase of rush VIT (end) were 27·5, 23·3, 25·6, 224·9, and 58·8 for total mononuclear cells, T cells, CD4+ T cells, CD8+ T cells and NK cells, respectively. T cells and NK cells showed statistically significant decreases ($P < 0{\cdot}05$).

The yield of PBMC per mL of blood was also significantly reduced immediately after the rush induction. This recovered to within the normal range by 1 month. We looked to see if a specific subset of cells was underrepresented in the peripheral blood after rush VIT, by specific leucocyte and subset counts. However, it appeared that all examined cells represented in the mononuclear compartment (monocytes, CD4+ T cells, CD8+ T cells, NK cells and total mononuclear cells) were reduced by about 25 % one day after rush induction (Fig. 4).

Over the period of rush immunotherapy (usually 24 h) there was a significant ($P < 0{\cdot}01$) reduction in the amount of IL-4 subsequently produced in culture when PBMC were stimulated with bee venom (Fig. 5). IL-4 production recovered to pre-treatment levels at 3 weeks, and fell progressively thereafter. Venom specific IFNγ production was induced from 2 months. In conventional VIT, there was a progressive fall in IL-4 production to venom, significant after 2 months, and undetectable by 6 months. After 2–3 months' treatment, marked IFNγ production to venom occurred. Although IFNγ production remained switched on thereafter, levels were less well maintained than after rush VIT.

Discussion

This study shows that untreated bee venom allergic patients have a TH-2 (IL-4) response to allergen (bee venom) and a TH-1 (IFNγ) response to antigen (SK-SD). In contrast, controls had a null response (neither TH-1 or TH-2 cytokines) to bee venom and a normal TH-1 response to antigen. Patients were able to respond, as normal subjects, to mitogen, with both IFNγ and IL-4 production (data not shown). Both rush and conventional VIT resulted in changes in cytokine secretion confirming a preliminary report on a smaller number of patients [20]. The primary change appeared to be loss of the abnormal TH-2 response to bee venom. A TH-1 response to venom was induced, and this seemed to occur soon after the switch-off of IL-4 production. However, there was a striking difference in the pattern of cytokine change after rush or conventional VIT. One day after rush induction there was a significant fall in IL-4 production to bee venom, whereas in conventional VIT, IL-4 production gradually fell, a significant reduction occurring by 2–3 months. Since only nine patients were studied during VIT, these results must be regarded as preliminary.

The model used, stimulation of PBMC *in vitro* by

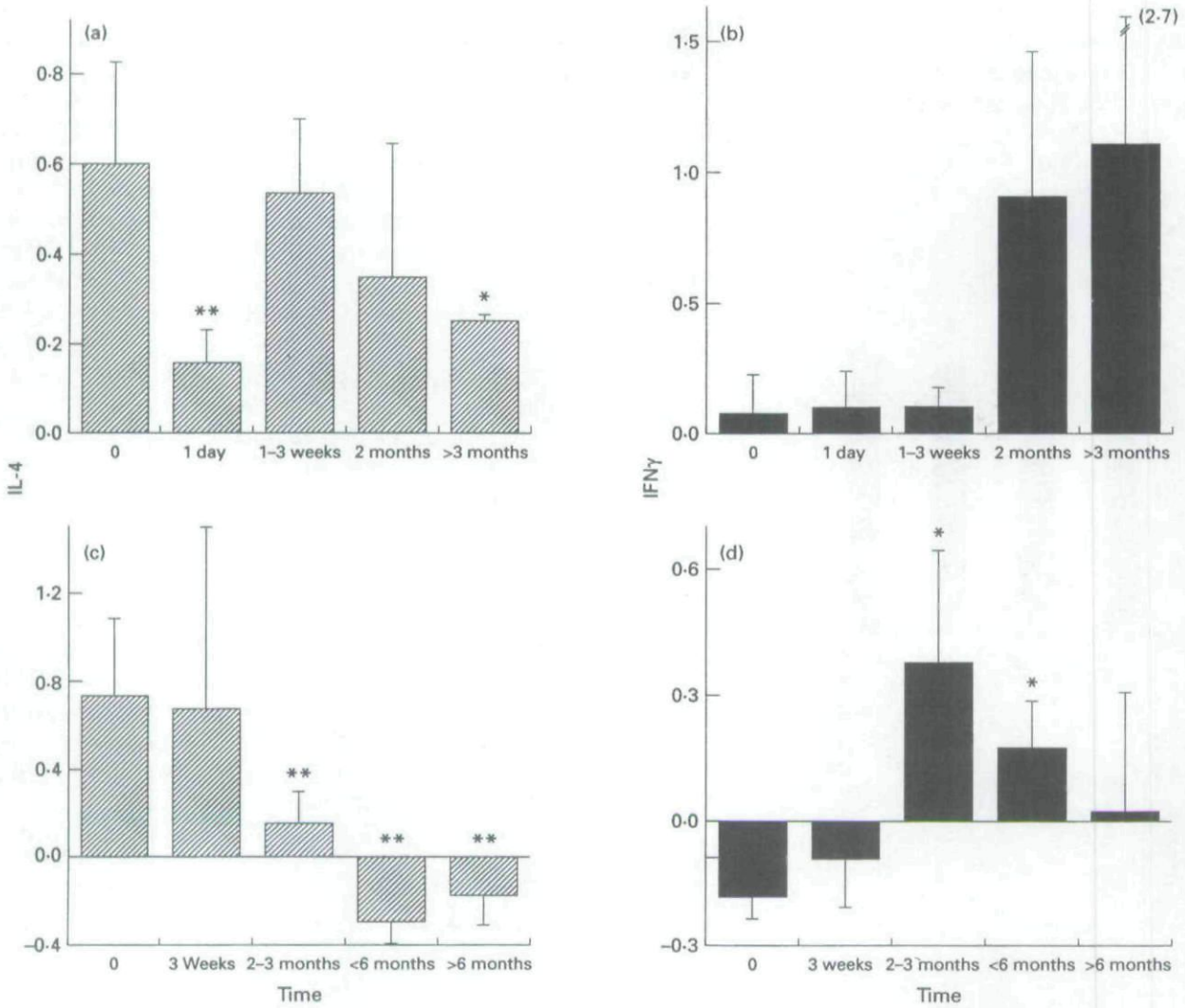

Fig. 5. Cytokine changes during rush and conventional VIT. IL-4 (left) and IFNγ (right) production by PBMC in response to bee venom stimulation *in vitro* during rush ($n = 5$, top) and conventional VIT ($n = 4$, lower panel). Statistical significance from pre-IT levels is indicated : $P < 0{\cdot}01$ (**) or $P < 0{\cdot}05$ (*). The induction period of rush is represented as 1 day. Cytokine levels are expressed as a proportional change compared with unstimulated controls, in mean total cytokine production, calculated by area under the curve analysis.

allergen, antigen or mitogen, was previously validated in house dust mite allergy, where we showed that allergic patients had a TH-2 response to *D. pteronyssinus* and a TH-1 response to recall antigen [15–17], the same pattern found in studies with allergen- or antigen-specific T cell clones [12,14,21]. The development of sensitive assays has allowed detection of low levels of IL-4 and IFNγ. This system has the advantage over T cell clones of measuring the entire repertoire of allergen-specific T cell responses, and of allowing sequential studies to look at effects of therapy.

There is nothing to suggest endotoxin contamination which could cause cytokine changes. The same preparation (and batch) was used for treatment and for all *in vitro* studies, and cytokine production differed in untreated patients and controls, and then changed in patients during VIT. No patient had reactions attributable to endotoxin.

The question of interest is how (or whether) these cytokine changes are related to the mechanism of VIT. In the longer term, loss of IL-4 and induction of IFNγ production would lead to decreased venom IgE and increased IgG production. However, there is a considerable body of evidence showing that these parameters

correlate poorly with clinical desensitization [7–10]. Despite this, the possibility remains that more subtle changes in the balance between IgE and IgG antibodies to venom or to individual allergens in venom, may be important [22,23]. However, changes in antibody levels fail to explain rush VIT, where clinical desensitization is achieved in days.

The immediate drop in IL-4 production in rush VIT, if representative of all venom-specific T cells, suggests profound down-regulation in T cell responsiveness to allergen. The mechanism is unclear but this might be due to T cell anergy [24] or activation-induced apoptosis [25]. Changes in cytokine or chemokine production might have an early effect by down-regulating mast cell or basophil reactivity. This would fit with the timing of clinical desensitization. Several cytokines and chemokines are known to modulate mediator release *in vitro*. In mice IL-4 enhances mediator release from mast cells [26] and MCAF/MCP-1 is a potent inducer of histamine release from human basophils [27]. Other cytokines, e.g. IL-3 and chemokines have similar but less potent effects on basophils and mast cells [28,29]. Reduced production of these factors would down-regulate mast cell mediator release. Complex interactions are likely to occur *in vivo* and it is known that granulocyte-macrophage colony stimulating factor (GM-CSF), IL-5 and IL-3 prime basophils for histamine release induced by MCAF and other stimuli [28].

An alternative explanation for the early fall in venom induced IL-4 secretion is loss of allergen-specific TH-2 cells from the blood compartment because of recruitment into the multiple large late-phase cutaneous reactions (LPR) at sites of venom injection. This is a particular feature of rush VIT, where after 1 day's treatment there are at least 14 large cutaneous LPRs involving a considerable area of both arms. The fall in IL-4 secretion was mirrored by changes in the proliferative response to venom in rush VIT. In addition, the yield of PBMC per mL of blood was significantly reduced on day 2, suggesting loss (or change of density) of mononuclear cells. Peripheral blood counts and analysis of T cell subsets, monocytes and NK cells on day 2 showed a reduction of about 25% in all of these, with a significant fall in T cells. These observations are compatible with recruitment of inflammatory cells as well as venom specific TH-2 cells into the LPR. It is well established that activated T cells infiltrate allergen-induced late phase responses [30,31] and the inflammatory infiltrate and process in the cutaneous LPR has been delineated by Frew and Kay [32].

One of the initial events in VIT would be low dose antigen encountering mast cell IgE. In regulation of B cell triggering and tolerance it has been shown that signalling via surface Ig and Fc receptors is down regulated by prior exposure to low dose immune complexes. This occurs by a biochemical uncoupling of surface Ig receptors from their G proteins prior to the inositol polyphosphate signalling cascade, which would otherwise result in cell activation [33]. A similar observation concerning the effect of antigen dose on mast cell degranulation has been made [34], suggesting a possible mechanism of desensitization.

If venom-specific TH-2 cells were recruited into the cutaneous LPRs, they would be exposed to high concentrations of allergen locally. There is evidence from other systems to suggest that the dose of antigen can influence the TH phenotype, low dose antigen favouring TH-2 responses and high dose antigen favouring TH-1 responses. Bee venom phospholipase A_2 (PLA) can elicit different TH cytokine production from PLA-specific T cell clones depending on the dose of antigen used, low doses causing TH-2 (IL-4) and high dose antigen causing TH-1(IFNγ) cytokine production [35]. This phenomenon has been demonstrated in a system where keyhole limpet haemocyanin was used as the specific antigen [36] and in a murine system looking at T cell responses to peptide antigens. In the latter model it was suggested that the different doses required for priming TH-1 and TH-2 responses reflected different activation requirements of the two cell types, TH-1 cells requiring a high ligand density and TH-2 cells a low ligand density [37]. The high dose of venom locally in the skin in rush VIT may initiate the 'TH-2 to TH-1 switch', by favouring production of TH-1 cells from naive T cells.

At a local level, human mast cells and basophils are known to be important producers of IL-4 [38,39]. IL-4 release is maintained after degranulation and is thought to be a potentiating agent in the selection and development of antigen-specific T cells which infiltrate allergic inflammatory reactions [39]. Basophil derived IL-4 is unlikely to form a significant fraction of the IL-4 measured in our culture system, since basophils are likely to release cytokines immediately after exposure to allergen, and basophils would not have survived in culture to release IL-4 by the time the peak cytokine secretion occurred. However, basophil or mast cell IL-4 secretion may be very important *in vivo*. Its removal could be important in permitting TH-1 cell selection on subsequent antigen exposure.

There has been no satisfactory explanation for the mechanism of rush VIT. Although it has been suggested that it may be due to subclinical depletion of mast cell mediators, there is no evidence to support this and two pieces of evidence against. We have anecdotal evidence to show that this is not the case, in that patients have

developed anaphylaxis in response to the penultimate venom injection of a rush schedule [40]. Muller's group have shown that the skin test response to codeine phosphate is unchanged after rush VIT [41]. Both observations suggest that mast cell mediators were not depleted at the end of rush induction.

Our findings in untreated allergic patients are in keeping with studies with TCC showing allergen-specific TCC are of the TH-2 type and antigen-specific TCC are TH-1 in patients allergic to *D. pteronyssinus* [12,14]. They also fit with *in situ* hybridization studies demonstrating the expression of the TH-2 cytokine mRNA in nasal or bronchial biopsies of patients with rhinitis or asthma [42,43].

The only previous study of cytokines in immunotherapy was in pollen IT for hay fever [44]. Cytokine expression was measured in biopsies in the allergen-induced late-phase cutaneous reaction. In untreated patients IL-4 and IL-5 mRNA expression but not TH-1 cytokine expression was found. After 1 year of IT, IL-2 and IFNγ (TH-1) mRNA expression was induced, but there was no reduction in the TH-2 cytokine message. The induction of TH-1 cytokines is similar to our findings in VIT but the TH-2 responses differ. However, this study differs from ours in a number of ways: the allergen and route of sensitization differ, cytokine mRNA rather than the secreted product was measured and only a single time point after IT was studied. It has been shown that cytokine mRNA need not correlate with cytokine secretion so that TH-2 cells may still be present after IT, as suggested by the grass pollen study, but they may not be secreting cytokines in response to specific allergen.

Taken together, uncoupling of the immediate allergic reactivity associated with mast cell or basophil degranulation, followed by high dose and persistent allergen exposure which elicits a TH-1 cytokine response, could explain the observed rapid clinical efficacy and the cytokine profiles elaborated following rush VIT, and be consistent with the time course of clinical desensitization and cytokine production during conventional VIT. Maintenance of desensitization might then be a balance between mast cell/basophil reactivity and the emergence of TH-1 cytokine producing effector and memory T cells with the concomitant switch away from TH-2-induced IgE production, and the production of specific IgG subclasses.

Acknowledgement

This work was supported by the Medical Research Council and the East Anglian Regional Health Authority.

References

1 McHugh SM, Lavelle B, Kemeny DM, Patel S, Ewan PW. A placebo-controlled trial of immunotherapy with two extracts of Dermatophagoides pteronyssinus in allergic rhinitis, comparing clinical outcome with changes in antigen-specific IgE, IgG and IgG subclasses. J Allergy Clin Immunology 1990; 86:521–31.

2 Sobotka AK, Valentine MD, Ishizaka K, Lichtenstein LM. A measurement of IgG blocking antibodies: development and application of a radio-immunoassay. J Immunol 1976; 117:84–90.

3 Golden DBK, Meyers DE, Kagey-Sobotka A, Valentine MD, Lichtenstein LM. Clinical relevance of the venom-specific immunoglobulin G antibody level during immunotherapy. J Allergy Clin Immunol 1982; 69:489–93.

4 Lessof MH, Sobotka AK, Lichtenstein LM. Effects of passive antibody in bee venom anaphylaxis. Johns Hopkins Med J 1978; 142:1–7.

5 Müller UR, Morris T, Bischof M, Friedli H, Skarvil F. Combined active and passive immunotherapy in honeybee-sting allergy. J Allergy Clin Immunol 1986; 78:115–22.

6 Bousquet J, Fontez A, Aznar R, Robinet-Levy M, Michel FB. Combination of passive and active immunization in honeybee venom immunotherapy. J Allergy Clin Immunol 1987; 79:947–54.

7 Blaauw PJ, Smithuis LOMJ. The evaluation of the common diagnostic methods of hypersensitivity for bee and yellow jacket venom by means of an in-hospital insect sting. J Allergy Clin Immunol 1985; 75:556–62.

8 Kampelmacher MJ, van der Zwann JC. Provocation test with a living insect as a diagnostic tool in systemic reactions to bee and wasp venom: a prospective study with emphasis on the clinical aspects. Clin Allergy 1987; 17:317–27.

9 Müller U, Helbling A, Bischof M. Predictive value of venom-specific IgE, IgG and IgG subclass antibodies in patients on immunotherapy with honey bee venom. Allergy 1989; 44:412–8.

10 Ewan PW, Deighton J, Wilson AB, Lachmann PJ. Venom specific IgG antibodies in bee and wasp allergy: lack of correlation with protection from stings. Clin Exp Allergy 1993; 23, 647–60.

11 Mosmann TR, Cherwinski H, Bond MM, Giedari MA, Coffman RL. Two types of murine helper T cell clones: 1. Definition according to the profiles of lymphokine activities and secreted proteins. J Immunol 1986; 136:2348–57.

12 Wierenga EA, Snoek M, de Groot C, Chretien I, Bos JD, Jansen HM, Kapsenberg ML. Evidence for compartmentalisation of functional subsets of CD4+ T lymphocytes in atopic patients. J Immunol 1990; 144:4651–6.

13 Romagnani S. Human TH1 and TH2 subsets: doubt no more. Immunol Today 1991; 12:256–7.

14 Parronchi P, Macchia D, Piccinni M-P et al. Allergen- and bacterial antigen-specific T cell clones established from atopic donors show a different profile of cytokine production. Proc Natl Acad Sci USA 1991; 88:4538–42.

15 McHugh SM, Wilson AB, Deighton J, Lachmann PJ,

Ewan, P W. IL-2, IL-6 and IFN-γ profiles from peripheral blood mononuclear cells of house dust mite allergic patients: a role for IL-6 in allergic disease. Eur J Allergy Clin Immunol 1994; 49:751–9.

16 McHugh SM, Wilson AB, Deighton J, Lachmann PJ, Ewan PW. IL-6 dominates the T_H2 cytokine response by PBMC in atopic patients. BSACI Annual Meeting (1993), Book of Abstracts, p. 26.

17 McHugh SM, Lachmann PJ, Ewan PW. Peripheral blood mononuclear cells from house dust mite allergic patients produce IL-2 in response to specific allergen challenge. Clin Exp Allergy 1993; 23:137–44.

18 Wilson AB, McHugh SM, Deighton J, Ewan PW, Lachmann PJ. A competitive inhibition ELISA for the quantification of human interferon-gamma. J Immunol Methods 1993; 162:247–55.

19 Bland J M and Altman D G. Statistical methods for assessing agreement between two different methods of clinical assessment. Lancet 1986; 1:307–10.

20 McHugh SM, Wilson AB, Deighton J, Stewart AG, Ewan PW. Bee venom immunotherapy induces T_H2 to T_H1 cytokine switch. (abstract) Eur J Allergy Clin Immunol 1993; 48, (Suppl 16): 97.

21 Del Prete G, De Carli M, Mastromauro C et al. Purified protein derivative of Mycobacterium Tuberculosis and excretory-secretory antigen(s) of Toxocara canis expand in vitro human T cells with stable and opposite (type 1 helper or type 2 helper) profile of cytokine production. J Clin Invest 1991; 88:346–50.

22 Deighton J, Lachmann PJ, Ewan PW. Western blotting reveals the diversity of IgE and IgG response to bee venom antigens. BSACI Annual Meeting (1993), Book of Abstracts, p. 34.

23 Ewan PW, Deighton J, Lachmann PJ. An analysis of the IgE and IgG response to wasp venom antigens before and after venom immunotherapy. BSACI Annual Meeting (1993), Book of Abstracts, p. 34.

24 O'Hehir RE, Yssel H, Verma S, de Vries JE, Spits H, Lamb JR. Clonal analysis of differential lymphokine production in peptide and superantigen induced T cell anergy. Int Immunol 1991; 3:819–26.

25 Wesselborg S, Kabelitz D. Activation-driven death of human T cell clones: time course kinetics of the induction of cell shrinkage, DNA fragmentation, and cell death. Cell Immunol 1993; 148:234–41.

26 Coleman JW, Holliday MR, Kimber I, Zesbo KM, Galli SJ. Regulation of mouse peritoneal mast cell secretory function by stem cell factor, IL-3 or IL-4. J Immunol 1993; 150: 556–62.

27 Kuna P, Reddigari SR, Rucinski D, Oppenheim JJ, Kaplan AP. Monocyte chemotactic and activating factor is a potent histamine-releasing factor for human basophils. J Exp Med 1992; 175:489–93.

28 Okayama Y, Church MK. IL-3 primes and evokes histamine release from human basophils but not mast cells. Int Arch Allergy Immunol 1992; 99:343–5.

29 Bischoff SC, Dahinden CA. C-kit Ligand: A unique potentiator of mediator release by human lung mast cells. J Exp Med 1992; 175:237–49.

30 Bentley AM, Meng Q, Robinson DS et al. Increases in activated T lymphocytes, eosinophils and cytokine mRNA expression for interleukin-5 and granulocyte/macrophage colony stimulating factor in bronchial biopsies after allergen inhalation challenge in atopic asthmatics. Am J Respir Cell Mol Biol 1993; 8:35–42.

31 Jacobson MR, Varney V, Sudderick R et al. Immunohistology of allergen-induced late nasal responses. J Allergy Clin Immunol 1991; 87 (abstr.):304.

32 Frew AJ, Kay AB. The relationship between infiltrating CD4+ lymphocytes, activated eosinphils and the magnitude of the allergen-induced late-phase cutaneous reaction. J Immunol 1988; 141:4158–64.

33 Warner GL, Scott DW. A polyclonal model for B cell tolerance. I. Fc-dependent and Fc-independent induction of nonresponsiveness by pretreatment of normal splenic B cells with anti-Ig. J Immunol 1991; 146:2185–1.

34 Levi-Schaffer F, Gare M, Shalit M. Unresponsiveness of rat peritoneal mast cells to immunologic reactivation. J Immunol 1990; 145:3418–24.

35 Carballido JM, Carballido-Perig N, Terres G, Heusser CH, Blaser K. Bee venom phospholipase A_2-specific T cell clones from human allergic and non-allergic individuals: cytokine patterns change in response to antigen concentration. Eur J Immunol 1992; 22:1357–63.

36 DeKruyff RH, Fang Y, Umetsu DT. IL-4 synthesis by in vivo primed keyhole limpet haemocyanic-specific $CD4^+$ T cells. I. Influence of antigen concentration and antigen-presenting cell type. J Immunol 1992; 149:3468–76.

37 Pfeiffer C, Murray J, Madri J, Bottomly K. Selective activation of T_H1 and T_H2-like cells in vivo - response to human collagen IV. Immunol Revs 1991; 123:65–84.

38 Bradding P, Feather IH, Howarth PH et al. IL-4 is localised to and released by human mast cells. J Exp Med 1992; 176:1381–6.

39 MacGlashan D, White JM, Huang SK et al. Secretion of IL-4 from human basophils. The relationship between IL-4 mRNA and protein in resting and stimulated basophils. J Immunol 1994; 152:3006–16.

40 Ewan PW. Mechanism of allergen immunotherapy. Position paper on allergen immunotherapy: report of a BSACI working party. Ed Kay A.B. Clin Exp Allergy 1993; 23(Suppl 3):19–22.

41 Jutel M, Skarbic D, Pickler WJ, Muller UR. Ultra-rush bee venom immunotherapy does not result in exhaustion of skin mast cells but influences the T cell reactivity to specific allergen. Abstracts ICACI/EAAI Allergy and Clin Immunol News 1994; (Suppl 2):125.

42 Robinson D, Hamid Q, Bentley A et al. Activation of CD4+ T cells, increased T_H2-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. J Allergy Clin Immunol 1993; 92:313–24.

43 Durham SR, Ying S, Varney VA et al. Cytokine messenger

RNA expression for IL-3, IL-4, IL-5 and granulocyte/macrophage-colony-stimulating factor in the nasal mucosa after local allergen provocation: relationship to tissue eosinophils. J Immunol 1992; 148:2390–4.

44 Varney VA, Hamid Q, Gaga M et al. Effect of grass pollen immunotherapy on cytokine mRNA expression during allergen-induced late cutaneous responses. J Clin Invest 1993; 92:871, 644–51.